PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PCT-127	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/ES 03/00063	International filing date *(day/month/year)* 03.02.2003	Priority date *(day/month/year)* 04.02.2002
International Patent Classification (IPC) or both national classification and IPC C12P23/00, C12P23/00		
Applicant VITATENE, S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 12 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08.08.2003	14.06.2004
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/ES 03/00063

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-36 as originally filed

Claims, Numbers

1-27 filed with telefax on 11.05.2004

Drawings, Sheets

1-9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

   see separate sheet

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees, the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:

   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3-12,17,24
	No:	Claims	1,2,13-16,23-26
Inventive step (IS)	Yes:	Claims	5-12,17
	No:	Claims	1-4,13-16,18-26
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	-

2. Citations and explanations

see separate sheet

Re Item I
Basis of the Report

AMENDMENTS (Art. 34(2)(b) PCT).

B.1 The subject-matter of amended claim 1, which concerns a method for selecting *X. dendrorhous* strains, is only partially supported by the application as originally filed (see the description on: page 1, lines 5-7; page 8, lines 14-16 and 20-21; page 9, lines 19-22, 29-34 and 37-38). In particular, a generic "solid medium" containing inhibitors or compounds is referred to in the amended claim for the first selection step, whereas the supporting passage of the description specifically refers to a "YEPDA medium" (see page 9, lines 29-34). The new claim definition results therefore in a generalization of the original disclosure, due to the omission of the YEPDA feature in the claim.
The simple fact that the originally filed description discloses YEPDA as a solid medium (see page 9, lines 16-18) does not support this generalization.
This International Preliminary Examination Authority is therefore of the opinion that amended claim 1 relates to subject-matter which extends beyond the content of the application as originally filed because of this generalization.
The Applicant/Representative has not provided any further relevant comment concerning this generalization and the omission of the YEPDA feature in the claim.

B.2 In view of Rule 70.2(c) PCT and the substantial differences between the amended and the originally filed claims, the International Preliminary Examination Report has been based on the set of claims as originally filed.

Re Item IV
Lack of unity of invention

U.1 The common concept, which would link the claimed subject-matter together, is the idea of producing astaxanthin by culturing strains of *X. dendrorhous*.

U.1[a] This idea is not novel because the prior art discloses the production of astaxanthin by means of fermentation processes involving strains of *X. dendrorhous* (see for example points 1.5 and 1.6 below).

U.1[b] In addition, this idea does not appear to be novel over the fermentation processes carried out in the presence of *P. rhodozyma* (see for example points 1.1 and 1.2

below) because *P. rhodozyma* and *X. dendrorhous* are the names of the two sexual states of the same microorganism and the distinction between these two yeast forms does not apparently apply to fermentation processes for the production of astaxanthin (see point 1.8 below and the indifferent use of the two names in D5, D6 and D7). Moreover, strains of *P. rhodozyma* are to be considered transformed derivatives of *X. dendrorhous* as defined in the present application (see claim 1).

U.1[c] Hence, the concept above cannot be considered a general inventive concept according to Rule 13.1 PCT, and a lack of unity "a posteriori" is indicated.

U.2 With respect to the problem of improving the fermentative production of astaxanthin in the presence of *X. dendrorhous*, the present application provides the following different solutions:

U.2[a] supplementing duroquinone during the fermentation process (see claims 3-4);

U.2[b] supplementing retinal or trisporic acids for inducing carotenogenesis during the fermentation process (see claims 5-8);

U.2[c] supplementing glutamate during the fermentation process (see claims 9-10);

U.2[d] supplementing the fermentation medium of table I, line 5 (see claims 11-12);

U.2[e] illuminating the fermentation medium (see claims 13-17); and

U.2[f] seeding and cultivating the microorganisms according to the procedure of claim 18 (see claims 18-22).

U.3 Having regards to the technical features which clearly characterize the different solutions, there is no single technical relationship among these solutions involving one or more of said technical features, to which an inventive step could be addressed (Rule 13.2 PCT). Hence, each of these solutions relate to a separate invention or group of inventions.

U.3[a] The fact that all these solutions could account for the improved astaxanthin yield referred by claims 1 and 2 is not to be regarded as a clear technical feature limiting the claimed subject-matter. By reference to the minimum astaxanthin concentrations, these claims attempt a definition in terms of the result to be achieved, which merely amounts to a statement of the underlying problem (Art. 6 PCT). The technical features necessary for achieving the desired result are to be taken into account (see for example the list of solutions above).

U.4 The separate inventions or groups of inventions are:

U.4[a] Subject 1: claims 1-2(partially),3-4,13-26(partially).
Fermentation processes and products thereof characterized in that duroquinone is added during the fermentation process. Claims 1-2 and 13-26 are partially to be considered within this group in so far as they only involve the addition of duroquinone.

U.4[b] Subject 2: claims 1-2(partially),5-8,13-26(partially).
Fermentation processes and products thereof characterized in that retinal or trisporic acids is/are added during the fermentation process for inducing carotenogenesis. Claims 1-2 and 13-26 are partially to be considered within this group in so far as they only involve the addition of this/these carotenogenesis-inducing agent(s).

U.4[c] Subject 3: claims 1-2(partially),9-10,13-26(partially).
Fermentation processes and products thereof characterized in that glutamate is added during the fermentation process. Claims 1-2 and 13-26 are partially to be considered within this group in so far as they only involve the addition of glutamate.

U.4[d] Subject 4: claims 1-2(partially),11-12,13-26(partially).
Fermentation processes and products thereof characterized in that the specific culture medium of table I, line 5 is used for the fermentation process. Claims 1-2 and 13-26 are partially to be considered within this group in so far as they only involve the use of this culture medium.

U.4[e] Subject 5: claims 1-12(partially),13-17,18-26(partially).
Fermentation processes and products thereof characterized in that the fermentation medium is illuminated during the fermentation process. Claims 1-12 and 18-26 are partially to be considered within this group in so far as they only involve the illumination of the fermentation medium.

U.4[f] Subject 6: claims 1-17(partially),18-22,23-26(partially).
Fermentation processes and products thereof characterized by the specific procedure for seeding and cultivating the microorganisms as described in claim 18. Claims 1-17 and 23-26 are partially to be considered within this group in so far as they only involve this specific procedure.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. DOCUMENTS.

Reference is made to the following documents:

D1: WO94/23594 A (cited in the International Search Report);
D2: EP438182 A (cited in the International Search Report);
D3: ES 2115637 T (cited in the International Search Report);
D3': EP 551676 A (the corresponding European application);
D4: EP474347 A (cited in the International Search Report);
D5: Cruz J.M. & Parajo' J.C., *Food Chemistry* (1998) vol. 63, no. 4, pages 479-484;
D6: Vazquez M., *Food Technology and Biotechnology* (2001) vol. 39, no. 2, pages 123-128;
D7: Schroeder W.A. & Johnson E.A., *Journal of General Microbiology* (1993) vol. 139, pages 907-912;
D8: EP 1035206 A (cited in the application).

1.1 D1 discloses the production of astaxanthin by means of fermentation processes carried out in the presence of strains of *P. rhodozyma* (see abstract and example 2). In particular, the astaxanthin yield achieved by means of these fermentation processes is higher than 5000 ppm (see tables 1-3).

1.1[a] In addition, D1 discloses methods of increasing astaxanthin production by culturing *P. rhodozyma* strains under light irradiation (see example 6) and the use of UV light as a mutagenesis factor for the selection of the most efficient *P. rhodozyma* strains (see page 9, first paragraph and page 10, second paragraph).

1.2 D2 discloses fermentation methods for the production of astaxanthin by cultivating *P. rhodozyma* strains (see claims 9-11). These methods account for astaxanthin yields higher than 5000 ppm (see table 1, the last three lines).

1.2[a] In addition, D2 discloses the use of UV light for inducing mutations in the yeast strains (see page 3, lines 13-15).

1.3 D3, D3' and D4 disclose *P. rhodozyma* strains for use in the fermentative production of astaxanthin (see abstracts).

1.3[a] In particular, D3 (or D3') teaches that the astaxanthin concentration in the

fermentation medium is affected by the illumination conditions during fermentation (see page 2, lines 48-50).

1.3[b] D4 discloses the use of UV light as a mutagenesis factor for the selection of suitable yeast strains (see page 2, line 47).

1.5 D5 discloses a fermentation process for the production of astaxanthin by cultivating *X. dendrorhous* (see abstract).

1.6 D6 discloses the production of astaxanthin by a *X. dendrorhous* strain (see abstract). In particular, the astaxanthin production increases if the microorganism is grown in the light.

1.7 D7 discloses the increased production of astaxanthin by *P. rhodozyma* in the presence of duroquinone (see table 2).

1.8 D8 discloses processes for the production of astaxanthin involving enzymes derived from *P. rhodozyma* (see abstract and claims 9-13). In addition, D8 teaches that the names *P. rhodozyma* and *X. dendrorhous* are used to designate two sexual states of the same microorganism (see page 2, lines 14-16).

2. CLARITY (Art. 6 PCT).

2.1 Claims 1, 2, 12 and 19-22 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject-matter in terms of the result to be achieved, namely the minimum yield (concentration) of astaxanthin or biomass. In the present case, the reference to the desired result merely amounts to a statement of the underlying problem and the minimum yield is not to be regarded as a clear technical feature limiting the claimed subject-matter. The technical features necessary for achieving the desired result should be added to the definition of the claims (see for example dependent claims 3, 5, 7, 9, 11, 13-17 and 18).

3. NOVELTY (Art. 33(2) PCT) and INVENTIVE STEP (Art. 33(3) PCT).

3.1 The lack of clarity notwithstanding (see point 2.1 above), the subject-matter of independent claim 1 is not novel over D1 and D2 because these documents discloses fermentation processes, which result in the production of astaxanthin at high concentrations (>5000 ppm), and are carried out cultivating strains of *P. rhodozyma* (see points 1.1 and 1.2 above). These *P. rhodozyma* strains are to be considered mutants and/or transformed derivatives of *X. dendrorhous* as defined in the claim because *P. rhodozyma* and *X. dendrorhous* are the names of the two sexual states of the same microorganism (see points 1.8 above). Moreover, the distinction between these two yeast forms does not apparently apply to fermentation processes for the production of astaxanthin (see the indifferent use of the two names in D5, D6 and D7).

3.1[a] The subject-matter of independent claim 1 appears also to lack novelty over D5 and D6, which discloses methods for the production of astaxanthin by cultivating strains of *X. dendrorhous*, i.e. mutants according to the definition of claim 1 (see points 1.5 and 1.6 above). The indication of the desired astaxanthin yield of claim 1 is not to be regarded as a clear feature distinguishing the claimed subject-matter from the methods of D5 and D6 (see point 2.1 above).

3.2 Dependent claims 2-4, 13-16 and 18-22 do not appear to contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step, given the disclosure of the prior art.

3.2[a] In particular, the addition of duroquinone to the fermentation medium for increasing the astaxanthin production in cultures of *P. rhodozyma* is disclosed in D7 (see point 1.7 above).

3.2[b] The illumination of the yeast culture during fermentation has been already applied/described in the prior art for increasing astaxanthin production (see points 1.1[a], 1.3[a] and 1.6 above).

3.2[c] The specific seeding and culturing procedure of dependent claim 18 has not been disclosed in the available prior art, and therefore the subject-matter of claims 18-22 is considered to be novel. Nevertheless, it consists of procedural steps which come within the customary practice followed by the skilled person. The temperature conditions, seeding concentrations and time intervals disclosed/suggested in the prior art for cultures of *X. dendrorhous* and *P. rhodozyma* do not significantly differ from the ones defined in claim 18 (see D1-D7). Moreover, no unexpected effect (e.g. improved astaxanthin yield with respect

to D1-D7) is apparently achieved following this specific seeding and culturing procedure.

3.3 The subject-matter of claims 23, 25 and 26 is not novel over the fermentation cultures which are disclosed in the prior art and comprise *X. dendrorhous* or *P. rhodozyma* strains for the same reasons mentioned above (see point 3.1). D1 and D2 disclose the use of such a fermentation product in the food industry (see for example: D1, abstract and examples 10-11; D2, page 2, lines 11-20 and paragraph joining pages 5-6).

3.3[a] Dependent claim 24 does not appear to contain any features which, in combination with the features of claim 23 to which it refers, meet the requirements of the PCT in respect of novelty and/or inventive step, given the disclosure of D1 and D2.

3.4 The subject-matter of claims 5-12 and 17 appears to be novel and to involve an inventive step over the available prior art as explained below.

3.4[a] Following the reasoning of points 3.1 and 3.1[a] (see above), D1, D2, D5 and D6 can be independently considered to represent the relevant state of the art (see also points 1.1, 1.2, 1.5 and 1.6 above).

3.4[b] The problem to be solved can therefore be regarded as the provision of alternative and improved fermentation processes for the production of astaxanthin in the presence of yeast strains derived from *X. dendrorhous*.

3.4[c] The solutions proposed in dependent claims 5-12 and 17 consist in: (i) the addition of one substance among retinal, trisporic acids and glutamate during the fermentation process, or (ii) the use of the specific fermentation medium of table I, or (iii) the exposure of the fermentation medium to cycles of illumination/darkness.

3.4[d] These substances, fermentation medium and cycles of illumination/darkness distinguish the claimed subject-matter from the prior art. Hence, novelty is acknowledged.

3.4[e] Moreover, none of these solutions has been suggested in the prior art for solving the problem posed, and therefore inventive step is also acknowledged.

3.4[f] The subject-matter of these claims is to be considered as involving an inventive step also in the light of the unexpected and improved astaxanthin yield, which is achieved by applying any of these solutions (see examples 8-10 and figures 6, 7 and 8b of the present application). In particular, the cyclic illumination protocol of claim 17 account for a higher astaxanthin yield than the continuous illumination of the yeast cultures described in the prior art (see example 10 and figure 8b).

4. INDUSTRIAL APPLICABILITY (Art. 33(4) PCT).

4.1 Claims 1-26 relates to fermentation methods and products, which can be applied/made in the food industry, hence the subject-matter of these claims is to be considered industrially applicable according to article 33(4) PCT.

CLAIMS

1.- A method of obtaining new astaxanthin overproducing strains of *Xanthophyllomyces dendrorhous* (*X. dendrorhous*) consisting in inducing mutation of a parent strain of *X. dendrorhous* by incubating said parent strain under mutagenic conditions and by selecting the mutants obtained thereof, characterised because a first selection of astaxanthin overproducing mutants is achieved by growing the mutants on solid medium containing either inhibitors of the synthesis of steroids or compounds that alter the redox potential of the cell and then astaxanthin overproducing mutant strains were selected subsequently as a function of their yield on solid medium measured as:

i. deeper red coloration than the parent strain
ii. production of astaxanthin in darkness
iii. production of astaxanthin at temperatures over 20°C
iv. production of astaxanthin using sucrose as carbon source.

2.- A method according to claim 1 characterised by inducing mutation of the parent strain by incubating said strain in an appropriate culture medium containing a mutagenic agent selected among ethylmethanesulfonate (EMS) and N-methyl-N'-nitrosoguanidine (NTG) or irradiating said culture medium containing the parent strain of *X. dendrorhous* with ultraviolet arrays (UVA).

3.- A method according to any of the claims 1 or 2 in which the parent strain of *X. dendrorhous* was VKPM Y-2476.

4.- A method according to any of the claims 1-3 characterised by selecting the mutants by growing them in solid medium containing as inhibitor of the steroid synthesis a compound selected among β-ionone, imidazole,

diethylamine, 2-methylimidazole, nystatin and diphenylamine or, as compound that alter the redox potential of the cell, a compound selected among duroquinone or hydrogen peroxide.

5.- A method according to any of the claims 1-4 characterised by selecting the mutants by growing them in solid medium at 24°C.

6.- Astaxanthin overproducing mutants obtainable by the method of claims 1-5 characterised by possessing extrachromosomal elements consisting in linear double strand DNA plasmids and capable of producing at least 4000 ppm of astaxanthin after 6-7 days by flask fermentation.

7.- Astaxanthin overproducing mutants according to claim 6 characterised by producing at least 5000 ppm of astaxanthin after 7-9 days in industrial fermentation.

8.- Process for producing astaxanthin characterised in culturing in a suitable medium at appropriate growth conditions the mutants of claims 6 or 7 or derivatives thereof having the same extrachromosomal elements and having the same level of astaxanthin production.

9.- A process of fermentation according to claim 8, characterized in that duroquinone is added during the fermentation process.

10.- A process of fermentation according to claim 9, characterized in that duroquinone is added at a concentration of 25-50 µM.

11.- A process of fermentation according to claim 8, characterized in that retinal is added during the fermentation process.

12.- A process of fermentation according to claim 11, characterized in that retinal is added at a concentration of 35 µM.

13.- A process of fermentation according to claim 8, characterized in that trisporic acids are added during the fermentation process.

14.- A process of fermentation according to claim 13, characterized in that the trisporic acids are added at a concentration of 50-100 µg/ml.

15.- A process of fermentation according to claim 8, characterized in that glutamate is added during the fermentation process.

16.- A process of fermentation according to claim 15, characterized in that glutamate is added at a concentration of 5.5 mg/ml.

17.- A process of fermentation according to claim 8, characterized in that medium 5 described in Table I of the description is used for the fermentation process.

18.- A process of fermentation according to any of the claims 8-17, characterized in that the fermentation medium is illuminated during the fermentation process.

19.- A process of fermentation according to claim 18, characterized in that the source of illumination used is white light.

20.- A process of fermentation according to claim 18, characterized in that the source of illumination used is ultraviolet light.

21.- A process of fermentation according to any of the claims 18-20, characterized in that illumination is carried out from the start to the end of fermentation, preferably from 40 to 200 hours.

22.- A process of fermentation according to claim 21, characterized in that cycles of 6 hours of illumination / darkness are used.

23.- A process of fermentation according to any of the claims 8-22, characterized in that:

(a) Inocula of *X. dendrorhous* are seeded.

(b) The inocula of *X. dendrorhous* are cultivated for 48 hours at 20°C.

(c) Phases of primary culture of *X. dendrorhous* are seeded with about 0.4% (v/v) of the inoculum phase.

(d) The primary phases of *X. dendrorhous* are cultivated for 48-54 hours at 17-20°C.

(e) Each fermenter is seeded with 20% (v/v) of the primary phases of *X. dendrorhous*.

(f) The fermentation is incubated at 18-20°C for 60-72 hours and then at 17°C for 5-7 days.

24.- Biomass of *X. dendrorhous* with nutrient and pigmenting value, obtainable by the fermentation process described in claims 8 to 23, for use in food for humans and animals.

25.- Biomass according to claim 24, characterized in that it contains:

a) A concentration of at least 5000 µg/g of astaxanthin;

b) A concentration of at least 7400 µg/g of total carotenoids;

c) A concentration of at least 15% of proteins and

d) A concentration of at least 15% of carbohydrates.

26.- Compounds for animal food that consist of or contain the biomass of claims 24 and 25.

27.- Compounds for human food that consist of or contain the biomass of claims 24 and 25.